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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/716,054	11/17/2000	Gerald R. Crabtree	STAN-166	7611

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EXAMINER	
COOK, LISA V	

ART UNIT	PAPER NUMBER
1641	

DATE MAILED: 04/27/2006

Please find below and/or attached an Office communication concerning this application or proceeding.

Advisory Action Before the Filing of an Appeal Brief	Application No. 09/716,054	Applicant(s) CRABTREE ET AL.	
	Examiner Lisa V. Cook	Art Unit 1641	

--The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

THE REPLY FILED 18 November 2005 FAILS TO PLACE THIS APPLICATION IN CONDITION FOR ALLOWANCE.

1. ☒ The reply was filed after a final rejection, but prior to or on the same day as filing a Notice of Appeal. To avoid abandonment of this application, applicant must timely file one of the following replies: (1) an amendment, affidavit, or other evidence, which places the application in condition for allowance; (2) a Notice of Appeal (with appeal fee) in compliance with 37 CFR 41.31; or (3) a Request for Continued Examination (RCE) in compliance with 37 CFR 1.114. The reply must be filed within one of the following time periods:
   a) ☒ The period for reply expires *6* months from the mailing date of the final rejection.
   b) ☐ The period for reply expires on: (1) the mailing date of this Advisory Action, or (2) the date set forth in the final rejection, whichever is later. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of the final rejection.

   Examiner Note: If box 1 is checked, check either box (a) or (b). ONLY CHECK BOX (b) WHEN THE FIRST REPLY WAS FILED WITHIN TWO MONTHS OF THE FINAL REJECTION. See MPEP 706.07(f).

Extensions of time may be obtained under 37 CFR 1.136(a). The date on which the petition under 37 CFR 1.136(a) and the appropriate extension fee have been filed is the date for purposes of determining the period of extension and the corresponding amount of the fee. The appropriate extension fee under 37 CFR 1.17(a) is calculated from: (1) the expiration date of the shortened statutory period for reply originally set in the final Office action; or (2) as set forth in (b) above, if checked. Any reply received by the Office later than three months after the mailing date of the final rejection, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

NOTICE OF APPEAL

2. ☒ The Notice of Appeal was filed on *19 September 2005*. A brief in compliance with 37 CFR 41.37 must be filed within two months of the date of filing the Notice of Appeal (37 CFR 41.37(a)), or any extension thereof (37 CFR 41.37(e)), to avoid dismissal of the appeal. Since a Notice of Appeal has been filed, any reply must be filed within the time period set forth in 37 CFR 41.37(a).

AMENDMENTS

3. ☒ The proposed amendment(s) filed after a final rejection, but prior to the date of filing a brief, will not be entered because
   (a) ☒ They raise new issues that would require further consideration and/or search (see NOTE below);
   (b) ☐ They raise the issue of new matter (see NOTE below);
   (c) ☒ They are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal; and/or
   (d) ☐ They present additional claims without canceling a corresponding number of finally rejected claims.

   NOTE: *See Continuation Sheet*. (See 37 CFR 1.116 and 41.33(a)).
4. ☐ The amendments are not in compliance with 37 CFR 1.121. See attached Notice of Non-Compliant Amendment (PTOL-324).
5. ☐ Applicant's reply has overcome the following rejection(s): _____.
6. ☐ Newly proposed or amended claim(s) _____ would be allowable if submitted in a separate, timely filed amendment canceling the non-allowable claim(s).
7. ☒ For purposes of appeal, the proposed amendment(s): a) ☒ will not be entered, or b) ☐ will be entered and an explanation of how the new or amended claims would be rejected is provided below or appended.
   The status of the claim(s) is (or will be) as follows:
   Claim(s) allowed: *NONE*.
   Claim(s) objected to: *NONE*.
   Claim(s) rejected: *16-24 and 49-55*.
   Claim(s) withdrawn from consideration: *56-64*.

AFFIDAVIT OR OTHER EVIDENCE

8. ☐ The affidavit or other evidence filed after a final action, but before or on the date of filing a Notice of Appeal will not be entered because applicant failed to provide a showing of good and sufficient reasons why the affidavit or other evidence is necessary and was not earlier presented. See 37 CFR 1.116(e).
9. ☐ The affidavit or other evidence filed after the date of filing a Notice of Appeal, but prior to the date of filing a brief, will not be entered because the affidavit or other evidence failed to overcome all rejections under appeal and/or appellant fails to provide a showing a good and sufficient reasons why it is necessary and was not earlier presented. See 37 CFR 41.33(d)(1).
10. ☐ The affidavit or other evidence is entered. An explanation of the status of the claims after entry is below or attached.

REQUEST FOR RECONSIDERATION/OTHER

11. ☒ The request for reconsideration has been considered but does NOT place the application in condition for allowance because: attached.
12. ☐ Note the attached Information Disclosure Statement(s). (PTO/SB/08 or PTO-1449) Paper No(s). _____
13. ☒ Other: See Continuation Sheet.

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4/7/06

Continuation of 3. NOTE: The amendment filed 11/18/05 changed the claims from reading on in vivo procedures to in vitro analysis. This modification requires additional search and consideration with respect to the prior art. One applicable reference that will be applied to the claims include Briesewitz et al.(Proc. Natl. Acad. Sci, USA, March 1999, Vol.96, pg.1953-1958). A courtesy copy has been provided herewith for applicants review. The amendment also raises the issue of scope of enablement under 112, 1st paragraph. Accordingly, the amendment has not been entered .

Continuation of 13. Other: Briesewitz et al.(Proc. Natl. Acad. Sci, USA, March 1999, Vol.96, pg.1953-1958).

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4/7/06

Request for Reconsideration

1. Applicants response to the Final Office Action mailed April 19, 2005 and the interview on 18 November 2005 is acknowledged. Currently claims 16-24 and 40-64 are pending. Claims 56-64 are withdrawn from consideration as being directed to a non-elected invention. See 37 CFR 1.142(b) and MPEP § 821.03.

2. Currently claims 16-24 and 49-55 are under consideration.

Remarks

3. Applicants have elected to pursue claims 16-24 and 49-55 in the instant application. The rejections under final have been reconsidered and found to be applicable to claims 49-55. Accordingly the rejection is maintained and reiterated herein. There is no "new ground of rejection when the "basic thrust" of the rejection is the same. Ex parte Maas, 9 USPQ.2d 1746 (Bd. Pat. App. & Int. 1987).

REJECTIONS MAINTAINED

Claim Rejections - 35 USC § 112

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

4. Claims 16-24 and 49-55 are rejected under 35 U.S.C. 112, first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter, which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

Specifically claims 16-24 and 49-55 are drawn to a method of inhibiting a binding event in a host (*in vivo*) via the administration of an effective amount of a non-naturally occurring bifunctional inhibitor molecule.

Although the specification is enabling for the production and *in vitro* utility of non-naturally occurring bifunctional inhibitor molecules (See assay design and results - pages 20-21), it does not reasonably provide enablement for inhibiting protein-protein interactions *in vivo* with said non-naturally occurring bifunctional molecule.

Firstly, the development of non-naturally occurring/synthetic bifunctional molecules with binding characteristics of interest necessitates several conditions which have not been described in the instant specification.

In one instance, the prior art discloses that the development of inhibitors which can bind by both an active site specific interaction to a primary binding site and by a structure nonspecific hydrophobic interaction to a second site (bifunctional or bispecific molecules) requires several parameters to produce the intended binding specificity.

These parameters include; a crystal structure of the enzyme with the bound primary inhibitor, there must be a relatively "open" active site, to permit access to the active site, and the linker must introduce few unfavorable enthalpic and entropic interactions into the bound state. See Jein et al., J Med Chem., 1994, 37, 2100-2105, especially scheme 1 and page 2103, 2nd column 2nd paragraph. These parameters have not been addressed by the instant disclosure. Therefore one of skill in the art would not be able to predict the inhibition by binding of the claimed bifunctional molecule *in vivo*.

Secondly, the specification fails to teach the use of the claimed bifunctional inhibitor molecules in a living organism or host, such that an effective inhibition response is generated. The art has established that the successful production of bifunctional molecules and their utility in assay protocols does not predict their behavior in living animals or a host.

In other words, the bifunctional molecule must be evaluated in a host in order to determine efficacy or inhibition effects. See Kuduk et al. Bio & Med Chemistry Letters, 10, 2000, 1303-1306, in particular page 1305, 2nd column Conclusion, 2nd paragraph.

Further, the art teaches that successful *in vitro* bifunctional construct binding is not always indicative of the *in vivo* results exhibited by that same bifunctional molecule.

For example, see Peipp and Valerius page 510 – Conclusion, wherein "Results from clinical trials (in vivo effective dosage) with bispecific antibodies are less encouraging". Peipp and Valerius, Biochemistry Society Transactions, 2002, Volume 30, part 4, pages 507-511.

Accordingly, the specification does not provide substantive evidence that the claimed bifunctional molecules are capable of inhibiting a protein-binding event *in vivo*. This demonstration is required for the skilled artisan to be able to use the claimed bifunctional molecules for their intended purpose of preventing protein binding.

Without this demonstration, the skilled artisan would not be able to predict the outcome of the administration of the claimed bifunctional or bispecific non naturally occurring compositions. The ability to reasonably predict the capacity of a single non -naturally occurring bifunctional molecule to prevent protein-protein interaction in vivo is problematic.

Unfortunately, the art is replete with instances where even well characterized compositions that induce an in vitro response fails to elicit *in vivo* utility. See Waldmann, Science, Vol.252, 21 June 1991, pages 1657-1662, in particular page 1657 – 2nd column, wherein antibodies binding therapy has proven elusive and only one monoclonal antibody has been licensed for clinical utility. Accordingly, the art indicates that it would require undue experimentation to formulate and use a successful binding composition with out prior demonstration of efficacy.

Thirdly, *in vivo* testing or administration to a host entails considerations for host/patient tolerance, differences, validation, and monitoring; which are not set forth in the disclosure.

The disclosure merely outlines that the non-naturally occurring bifunctional molecule may be used to treat a variety of diseases, including cellular proliferation, autoimmune disease, cardiovascular diseases, hormonal abnormality, infectious disease, and the like without any supporting data/experimentation. See page 19 lines 24-35.

However, Tockman et al. (Cancer Research 52:2711s-2718s, 1992) teach considerations necessary for a suspected cancer antibody biomarker (intermediate end point marker) to have efficacy and success in a clinical application. See page 2716s. Although the reference is drawn to biomarkers for early lung cancer detection, the basic principles taught are clearly applicable to other compositions being administered and tracked in a host/patient.

Tockman teaches that prior to the successful application of newly described markers, research must validate the markers against acknowledged disease end points, establish quantitative criteria for marker presence/absence and confirm marker predictive value in prospective population trials, see abstract.

Early stage markers of carcinogenesis have clear biological plausibility as markers of preclinical cancer and **if validated** (emphasis added) can be used for population screening (p. 2713s, column 1). The reference further teaches that once selected, the sensitivity and specificity of the biomarker must be validated to a known (histology/cytology-confirmed) cancer outcome. The essential element of the validation of an early detection marker is the ability to test the marker on clinical material obtained from subjects monitored in advance of clinical cancer and *link* those marker results with subsequent histological confirmation of disease.

"This irrefutable link between antecedent marker and subsequent acknowledged disease is the essence of a valid intermediate end point [marker]", see page 2714s, column 1, Biomarker Validation against Acknowledged Disease End Points section. Clearly, prior to the successful application of newly described markers, markers must be validated against acknowledged disease end points and the marker predictive value must be confirmed in prospective population trials, see page 2716s, column 2, Summary section.

Tockman reiterates that the predictability of the art in regards to cancer prognosis and the estimation of life expectancies within a population with a disease or disorder are highly speculative and unpredictable. It has been set forth above that 1) the experimentation required to generate a non-naturally occurring bi-functional molecule which provides binding inhibition such that it would prevent target binding in a living host would be great as 2) there are no immunological experiments provided to demonstrate that the claimed bifunctional compositions are capable of mounting an efficient inhibition response and, more importantly, there are no challenge experiments to demonstrate that a person immunized with any one of the claimed compositions would be treated/protected from a disease.

There are no protocols provided which demonstrate which bifunctional molecules would be effective in immunization, nor are there any protocols detailing the amount of the bifunctional compositions needed to inhibit protein-protein interaction or mount a sufficient immune response in disease treatment, 3) there are no working examples provided in the instant specification, 4) the nature of the invention is a method for producing a non-naturally occurring bifunctional molecule which would provide binding inhibition and treatment in a host, 5) the relevant skill of those in the art is high yet 6) the state of the prior art has been shown to be highly unpredictable as evidenced by prior art afore mentioned, and lastly 7) the claims broadly encompass the administration of compositions to a host (in vivo) to target protein prevent binding in the host, it is therefore set forth that one of skill in the art could not make and/or use the invention without undue experimentation.

Based on the analysis and the teachings presented above it would require undue experimentation for the skilled artisan to practice this invention because there is no support in the specification for the enablement of the broadly claimed invention.

Therefore, in view of the insufficient guidance in the specification, extensive experimentation would be required to enable the claims and to practice the invention as claimed.

5. For reasons aforementioned, no claims are allowed.

6. Papers related to this application may be submitted to Group 1600 by facsimile transmission. Papers should be faxed to Group 1600 via the PTO Fax Center located in Crystal Mall 1.

The faxing of such papers must conform to the notice published in the Official Gazette, 1096 OG 30 (November 15, 1989). The Group 1641 – Central Fax number is (703) 872-9306, which is able to receive transmissions 24 hours/day, 7 days/week. In the event Applicant would like to fax an unofficial communication, the Examiner should be contacted for the appropriate Right Fax number.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Lisa V. Cook whose telephone number is (571) 272-0816. The examiner can normally be reached on Monday - Friday from 7:00 AM - 4:00 PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Long Le, can be reached on (571) 272-0823.

Any inquiry of a general nature or relating to the status of this application should be directed to the Group receptionist whose telephone number is (703) 308-0196.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see httpr//pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

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Biochemistry

Affinity modulation of small-molecule ligands by borrowing endogenous protein surfaces

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Contributed by Gerald R. Crabtree, December 16, 1998

ABSTRACT **A general strategy is described for improving the binding properties of small-molecule ligands to protein targets. A bifunctional molecule is created by chemically linking a ligand of interest to another small molecule that binds tightly to a second protein. When the ligand of interest is presented to the target protein by the second protein, additional protein–protein interactions outside of the ligand-binding sites serve either to increase or decrease the affinity of the binding event. We have applied this approach to an intractable target, the SH2 domain, and demonstrate a 3-fold enhancement over the natural peptide. This approach provides a way to modulate the potency and specificity of biologically active compounds.**

In nature, certain small-molecule ligands use a remarkable mechanism to enhance the affinity for their targets. These ligands bind to an endogenous protein, forming a new composite surface, which can bind then to a target protein. Presentation of the ligand by the larger endogenous protein vastly enlarges the surface area available for interactions with the target, facilitating additional protein–protein interactions and improved affinity.

For example, the peptide ligands of the T cell receptor use this strategy of surface enlargement to promote a high-affinity-binding event. By themselves, peptides usually have only a very low affinity for the polymorphic T cell receptor (TCR). For a high-affinity-binding event to occur, a peptide must be presented by the major histocompatibility complex (MHC). As the crystal structures of the trimeric TCR–peptide–MHC complex have shown, the TCR makes contacts not only with the peptide but also with the MHC (1, 2). The TCR-MHC contacts are important because mutations of these sites abolish the immune response and much of the remarkable specificity of the immune response is a result of this simple trimeric complex (3–5).

Certain microorganisms also make use of endogenous proteins to present and enhance the activity of their toxins. For example, the immunosuppressive drugs cyclosporin and FK506 are presented by cyclophilin and human FK506-binding protein 12 (FKBP), respectively, to inhibit the activity of a common protein target, calcineurin (6). By themselves, FK506 and cyclosporin have no measurable affinity for calcineurin. However, the FK506–FKBP and cyclosporin–cyclophilin complexes bind to calcineurin with high affinity (7). The cocrystal structure of calcineurin–FK506–FKBP reveals that the protein surfaces of calcineurin and FKBP make extensive contacts that promote the high affinity of the binding event (8, 9).

However, extensive protein–protein interactions between a presenting protein and the drug target are not always required to enhance the affinity of a ligand for its target protein. Like FK506, the macrolide drug rapamycin has no measurable affinity for its target, the cell cycle control protein FRAP. Instead, rapamycin forms a complex with FKBP to create a composite surface that binds to FRAP with high affinity. But, unlike the FK506–FKBP complex, the rapamycin–FKBP complex establishes only few contacts with its target (10).

Inspired by these natural examples, we envisioned that the specificity and affinity of a ligand–protein interaction or a drug–protein interaction could be modulated deliberately by borrowing additional surface contacts from an endogenous protein. Thus, we set out to chemically link a ligand for an abundant presenting protein to a weak binder for a target protein. By using chemical linkers of different lengths, tilt, and rotation, one may expect the resulting protein–protein interactions to be favorable or unfavorable so that binding occurs with enhanced or decreased affinity, respectively. As an extension of traditional medicinal chemistry, such an approach may be useful to modulate the potency and specificity of biologically active compounds.

As a first attempt to explore the feasibility of this approach, we selected two members of the FK506-binding protein family, FKBP12 and FKBP52, as presenter proteins. We chose the SH2 domain of the Fyn tyrosine kinase as the target protein because of its well established structure and the availability of ligands for SH2 domains (11). SH2 domains bind to peptides and proteins that contain phosphotyrosine residues, and they are commonly found in signaling proteins that regulate cell growth and differentiation. Ligands that bind to SH2 domains have been explored as possible therapeutics for cancer, osteoporosis, and inflammation and as immunosuppressive agents (12, 13). However, the development of ligands that bind to SH2 domains with high affinity and selectivity has met with little success, and SH2 domains generally are considered to be good examples of an intractable drug target.

MATERIALS AND METHODS

Synthetic Chemistry. Peptides were synthesized by using conventional solid-phase peptide synthesis methods. The FK506-derived mixed carbonate was synthesized as described by Spencer *et al.* (14). It was dissolved in dimethylformamide with triethylamine and a 2-fold excess of phosphotyrosyl-glutamyl-glutamyl-isoleucine (pYEEI). The coupled product was treated with hydrogen fluoride in acetonitrile to remove the two silyl ether protecting groups, and the desired product was purified by using reverse-phase HPLC. Synthetic ligand for FKBP (SLF) was synthesized according to the procedures of Holt *et al.* (15). SLF was coupled to the N terminus of the resin-bound protected pYEEI peptide by using PyBOP. The bifunctional SLFpYEEI was simultaneously deprotected and cleaved from the Novasyn TGT resin (Calbiochem) by using 25% trifluoroacetic acid and 2.5% triisopropylsilane in meth-

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Abbreviations: FKBP, human FK506-binding protein 12; pYEEI, tetrapeptide phosphotyrosyl-glutamyl-glutamyl-isoleucine; FKpYEEI, FK506 covalently linked to the peptide pYEEI; SLF, synthetic ligand for FKBP; SLFpYEEI, SLF covalently linked to pYEEI; GST, glutathione *S*-transferase; ITC, isothermal titration calorimetry; TCR, T cell receptor.

A Commentary on this article begins on page 1826.

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ylene chloride, and the desired product was isolated by using reverse-phase HPLC.

Peptide Coupling to Beads. The peptide pYEEI (1 mg) was dissolved in 1 ml of dimethyl sulfoxide and incubated with 1 ml of Affi-Gel 10 beads (Bio-Rad) for 6 hr at room temperature. The reaction was stopped by incubating the beads in 5 ml of ethanolamine (1 M, pH 8.0) for 1 hr. The beads were washed and resuspended 1:2 in 20 mM Tris, pH 7.2/150 mM NaCl.

Protein Expression. The human Fyn SH2 domain (residues 102–205, SIQA-LVVP) and human FKBP12 were expressed as glutathione *S*-transferase (GST) fusion proteins in *Escherichia coli*. The cDNAs were cloned into the pGEX2TK expression vector (Pharmacia). When required, the recombinant proteins were labeled with [γ-^{32}P]ATP on glutathione beads (Pharmacia) using protein kinase A (PKA) and the pGEX2TK-derived PKA site at the N terminus of FKBP12 and the Fyn SH2 domain. FKBP12 and the Fyn SH2 domain were cleaved from GST by using thrombin (Sigma) and the thrombin cleavage site between the PKA site and GST. Human FKBP52 was expressed in pET28c (Novagen) with a His tag at the N terminus. The recombinant protein was purified with Ni^{2+} nitrilotriacetic acid agarose beads (Qiagen).

Isothermal Titration Calorimetry. The binding constants of FKBP12, FKBP52, and the Fyn SH2 domain for SLF, SLF-pYEEI, and FKpYEEI were determined by using an Omega Isothermal Titration Calorimeter (Microcal, Northampton, MA). Protein in aqueous buffer (20–60 μM: 150 mM NaCl/20 mM Tris, pH 7.2) was equilibrated in the microcalorimeter cell at 25°C for 1–2 hr after being degassed for 10 min. Ligand (170–600 μM) in identical buffer as protein was taken into a 250-μl syringe after being degassed for 10 min. The syringe was loaded into the cell and spun at 400 rpm, and the system was allowed to equilibrate for 1–2 hr. Ligand was then injected into the cell (25 × 10 μl, 6-min intervals), and the heat evolved was quantitated. Binding constants were calculated from a numerical fit to the experimental data as described in ref. 16.

RESULTS

Molecular Design. Beginning with a tetrapeptide that binds to the Fyn SH2 domain, pYEEI (17), we synthesized two bifunctional molecules. Both molecules are capable of simultaneously binding to FKBP and the Fyn SH2 domain. The pYEEI peptide was linked covalently to two FKBP ligands, FK506 and SLF (15), to provide the desired bifunctional molecules, FKpYEEI and SLFpYEEI (Fig. 1). SLF is smaller than FK506 and does not project as far from the FKBP protein surface. By using the three-dimensional structures of FKBP12 (15, 18), FKBP52 (19), and the Fyn SH2 domain (20), the linkers between the two halves of the bifunctional molecules were designed to bring the FKBP surface into close proximity to the SH2 domain surface. The affinities of FKpYEEI and SLFpYEEI for recombinant FKBP12, FKBP52, and the Fyn SH2 domain were measured by using isothermal titration calorimetry (ITC, Table 1).

Binding Assay. To determine whether the bifunctional molecules allow the formation of a trimeric complex between Fyn and FKBP, GST-Fyn SH2 domain fusion proteins bound to glutathione beads were incubated with radioactively labeled FKBP12 and increasing concentrations of FKpYEEI or SLF-pYEEI (Fig. 2). After equilibrium was established, the beads were sedimented and the radioactively labeled FKBP12 associated with the beads was quantified. Both FKpYEEI and SLFpYEEI support the formation of a trimeric complex between the Fyn SH2 domain and FKBP12. However, FKpYEEI forms the trimeric complex more efficiently. FKpYEEI and SLFpYEEI can also form a trimeric complex between immobilized FKBP52 and the radioactively labeled Fyn SH2 domain (data not shown).

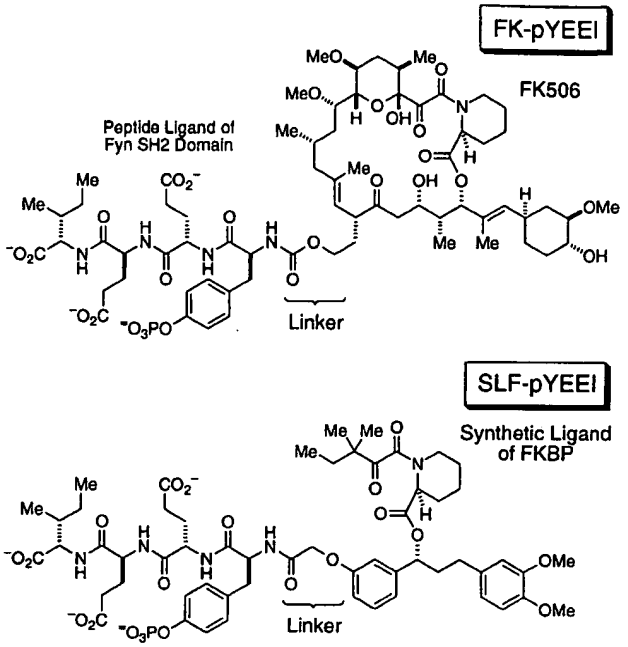

FIG. 1. Structures of FKpYEEI and SLFpYEEI. The C21 allyl group of FK506, which is required for calcineurin binding, was used to link the pYEEI peptide, effectively eliminating any immunosuppressive activity of the FKpYEEI molecule.

A competition binding assay was used to determine whether the pYEEI peptide bound more tightly to the Fyn SH2 domain when presented by FKBP (Fig. 3*A*). The pYEEI tetrapeptide was covalently attached to beads. The beads were incubated with radioactively labeled Fyn SH2 domain, and the bound protein was quantified by centrifuging the beads and counting the associated radioactivity. When FKpYEEI or SLFpYEEI was added to the binding reaction, the pYEEI peptide of the bifunctional molecules competed with the peptide beads for binding to the ^{32}P-labeled Fyn SH2 domain. As a consequence of this competition, less ^{32}P-labeled Fyn SH2 domain bound to the beads. Hence, low levels of radioactivity bound to the beads reflect high occupancy of the Fyn SH2 domain by a competing ligand in the solution (Fig. 3*B*).

Using a Borrowed Protein Surface to Enhance Affinity. By using this assay, FKBP12 or FKBP52 was added at various concentrations to a binding reaction to form a complex with FKpYEEI or SLFpYEEI. When presented by FKBP12, FK-pYEEI bound to the Fyn SH2 domain as well as free FKpYEEI (data not shown). However, the FKpYEEI–FKBP52 complex competed more effectively for binding to the Fyn SH2 domain than did FKpYEEI alone (Fig. 3*B*). To confirm that the observed effect depends on binding of FKpYEEI to the FK506-binding pocket of FKBP52, FK506 was added to the binding reaction. FK506 binds more tightly to FKBP12 and FKBP52 than either bifunctional molecule (Table 1). In the binding assay, as FK506 reaches an equimolar concentration

Table 1. Dissociation constants (K_d) for protein–ligand combination

	FKpYEEI, nM	SLFpYEEI, nM	FK506, nM	SLF, nM
FKBP12	45	60	0.4 (refs. 28 and 29)	20
FKBP52	150	5,000	50 (ref. 30 and 31)	3,000
Fyn SH2 domain	340	180	ND	ND

ND, not determined.

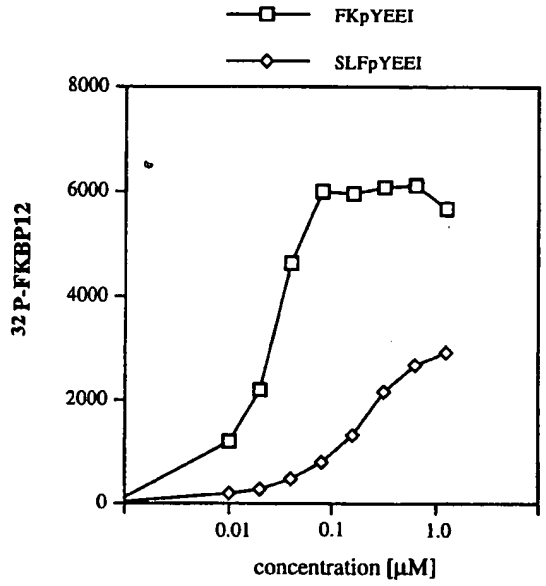

FIG. 2. FKpYEEI and SLFpYEEI form trimeric complexes with the Fyn SH2 domain and FKBP12. Recombinant GST-Fyn SH2 domain was expressed, bound to glutathione beads (7.5 μl beads, 0.55 nmol protein), and was incubated with 100 nM ^{32}P-labeled FKBP12 (10,000 cpm) in 100 μl of binding buffer (20 mM Tris, pH 7.2/150 mM NaCl). Increasing concentrations of FKpYEEI or SLFpYEEI were added and the reactions were rotated for 2 hr. The binding reactions were centrifuged in a pierced PCR tube to separate the beads from the supernatant. The radioactive protein associated with the beads was resuspended in 100 μl of PBS, which was added to EcoLite scintillation fluid (ICN) and counted in an LS5000CE liquid scintillation counter (Beckman).

with respect to FKBP52, most of the FKBP52 is bound to FK506. Under these conditions, the affinity-enhancing effect of FKBP52 was abolished (Fig. 3*C*). These results demonstrate that the enhanced affinity of FKpYEEI for the Fyn SH2 domain depends on FKpYEEI binding to the FK506-binding pocket of FKBP52.

To quantify the increase in affinity of the Fyn SH2 domain for pYEEI presented by FKBP52, we measured the binding of the ^{32}P-labeled Fyn SH2 domain to peptide beads as a function of the concentration of FKpYEEI (Fig. 3*D*). FKBP52 enhances the affinity by a factor of 3, which is reflected in a shift of the IC_{50} from 750 nM in the absence of FKBP52 to an IC_{50} of 250 nM in the presence of FKBP52. ITC measurements confirmed the approximately 3-fold affinity enhancement (FKpYEEI plus Fyn SH2 domain: K_d = 340 nM versus the FKpYEEI–FKBP52 complex plus Fyn SH2 domain: K_d = 120 nM).

We considered the possibility that FKBP12 and FKBP52 present the pYEEI peptide in different orientations. However, the structures of the FK506-binding domains from both proteins are very similar (19), and it is unlikely that preorganization of the relatively flexible pYEEI peptide is responsible for the observed binding enhancement. Thus, we conclude that when the Fyn SH2 domain binds to the FKpYEEI-FKBP52 complex, the SH2 domain makes additional favorable protein–protein interactions with FKBP52 that enhance the overall stability of the trimeric complex (Fig. 3*E*).

Using a Borrowed Protein Surface to Reduce Affinity. In contrast to the FKpYEEI–FKBP52 complex, the SLFpYEEI–FKBP12 complex displays decreased affinity for the Fyn SH2 domain. In the competition binding assay, greater amounts of the Fyn SH2 domain bind to the peptide beads in response to increasing concentrations of FKBP12 (Fig. 4*A*). This result indicates that the FKBP12–SLFpYEEI complex is a less effective competitor than SLFpYEEI alone. The effect is reversed by the addition of FK506, which indicates that SLFpYEEI binds to the FK506-binding pocket of FKBP12 (Fig. 4*B*). The IC_{50} of SLFpYEEI for the Fyn SH2 domain when presented by FKBP12 was increased 6-fold from 0.25 to 1.5 μM (Fig. 4*C*). This increase was confirmed by ITC (SLFpYEEI plus Fyn SH2 domain: K_d = 180 nM versus the SLFpYEEI–FKBP12 complex plus Fyn SH2 domain: K_d = 1.0 μM; 5.5-fold increase). We interpret this decrease in affinity to indicate that the FKBP12 surface establishes unfavorable interactions with the Fyn SH2 domain surface in the trimeric complex (Fig. 4*D*). The SLFpYEEI–FKBP52 complex does not affect the binding of the Fyn SH2 domain to pYEEI (data not shown). Considering the four possible complexes for presentation of the pYEEI peptide (two FKBPs and two bifunctional molecules), one complex improves binding of the pYEEI peptide to the Fyn SH2 domain, one complex diminishes binding, and two complexes have no measurable effect.

DISCUSSION

Borrowing Endogenous Proteins to Enhance the Characteristics of Small-Molecule Ligands. Our findings demonstrate that a phosphopeptide ligand for the Fyn SH2 domain can be engineered to bind more tightly to its protein target by inducing the formation of a trimeric complex. In the case of FKBP52, FKpYEEI, and the Fyn SH2 domain, the trimeric complex is more stable than would be expected based on the stabilities of individual bimolecular complexes (Table 1). Although our data do not definitively demonstrate new protein–protein interactions resulting from the covalent linkage of FK506 to the pYEEI peptide, the observation that the change in affinity is related to both the structure of the ligand (SLF vs. FK506) and the borrowed endogenous protein (FKBP12 vs. FKBP52) points to the surface between the SH2 domain and the FKBP as the origin of the altered affinity.

One explanation for the increase in affinity is that additional protein–protein interactions between FKBP52 and the SH2 domain surface make a significant, direct energetic contribution to the stability of the complex. Alternatively, the additional distal interactions may indirectly enhance the free energy of binding of the peptide ligand to the SH2 domain. This possibility is based on the analysis of the energetic contributions of single amino acid side chains to protein–protein interactions. The area of contact between two proteins is often large and flat (21, 22), but, interestingly, a major part of the free energy of binding of two interacting protein surfaces can be contributed by a limited number of clustered amino acids. These clusters have been designated as "hot spots" (23), and the surrounding contacts may serve to insulate the critical amino acids from bulk solvent. Thus, the SH2 domain-FKBP52 contacts may limit the accessibility of water to pYEEI and the binding pocket so that their energetic interactions are increased. Ultimately, structural studies will be helpful for confirming the role of FKBP-SH2 domain contacts in the affinity modification demonstrated here.

Plasticity of Binding Surfaces. The establishment of favorable or, at least, nondetrimental contacts will depend on the juxtaposition of the presenter protein surface to the surface of the target protein. Recent studies of human growth hormone and the TCR suggest that the plasticity of protein surfaces can act favorably in the attempt to establish beneficial protein–protein contacts between the presenter protein and the target. Mutations in growth hormone that compensate for a mutation in the core region of its receptor have been shown to lead to major rearrangements at the protein–protein interface (24). The crystal structure of the mutant proteins reveals that amino acid side chains reorganize to establish new interactions or to avoid unfavorable interactions. The plasticity of protein surfaces is also apparent in the binding of the TCR to peptide–

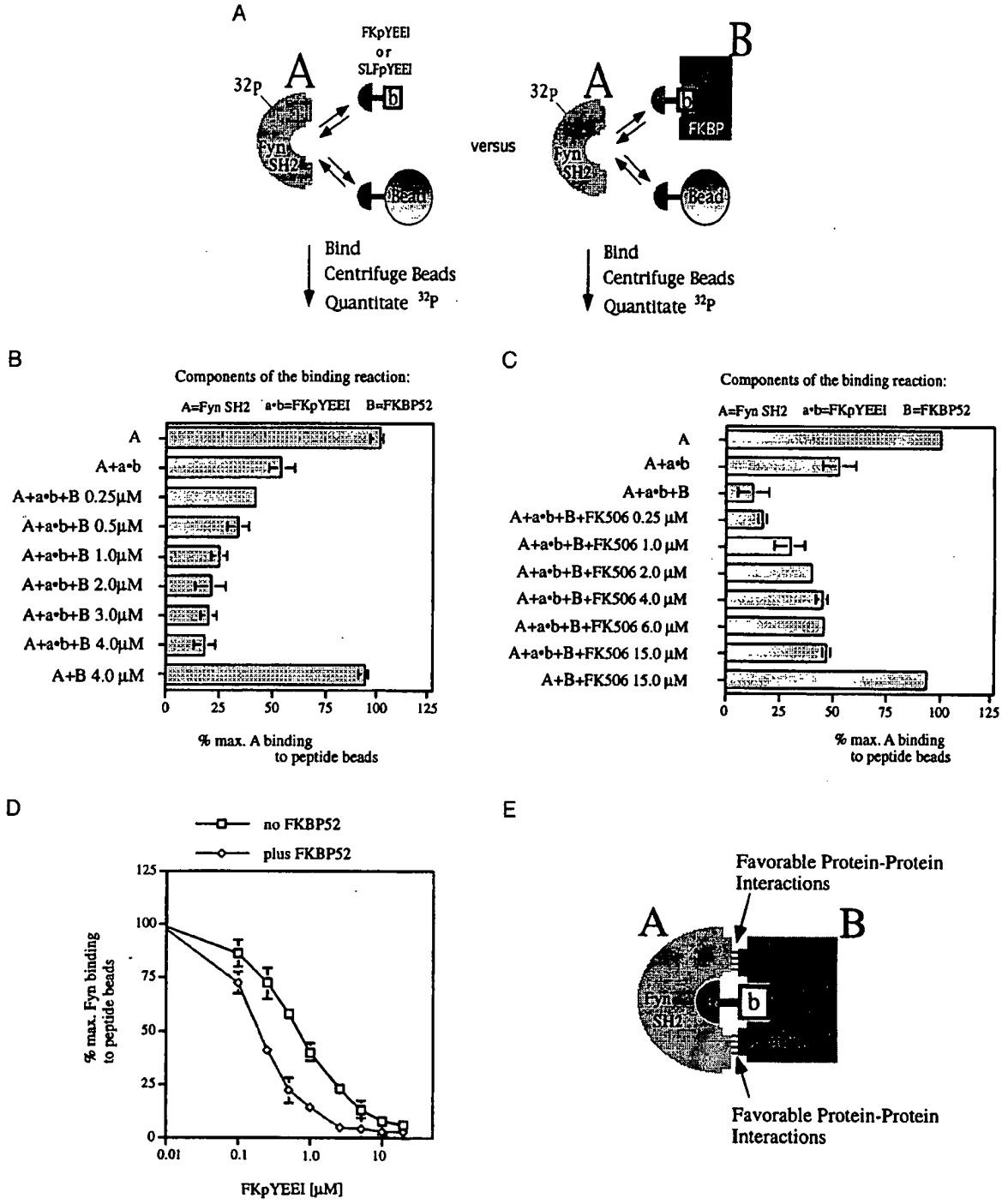

FIG. 3. Fyn SH2 domain binding to FKpYEEI alone or in a complex with FKBP52. (*A*) Model of the competition binding assay. (*B–D*) In 100 μl of binding buffer, peptide beads (7.5 μl) were incubated with the ^{32}P-labeled Fyn SH2 domain (200 nM, 14,000 cpm) and the molecules stated below. After a 2-hr incubation time, the beads were centrifuged and the radioactive Fyn SH2 domain bound was quantitated. (*B*) Addition of FKpYEEI (1.0 μM) and increasing concentrations of FKBP52 (0.25–4.0 μM). (*C*) Addition of FKpYEEI (1.0 μM), FKBP52 (2.0 μM), and increasing concentrations of FK506 (0.25–15 μM). The maximal radioactivity associated with the beads (=100%) was 9,139 cpm for *b* and 10,467 cpm for *C*. (*D*) Competition binding curves: ^{32}P-labeled Fyn SH2 domain (200 nM, 16,000 cpm) was incubated without or with FKBP52 (4.5 μM) in 100 μl of binding buffer. Increasing concentrations of FKpYEEI (0.1–20.0 μM) were added to the binding reactions. The maximal radioactivity associated with the beads was 12,569 cpm (=100%) for the ^{32}P-labeled Fyn SH2 domain alone and 10,250 cpm (=100%) for the ^{32}P-labeled Fyn SH2 domain in the presence of FKBP52. All data points were taken in triplicate, and the average is plotted. (Bars = SE.) (*E*) Model for favorable, affinity-enhancing protein–protein interactions.

MHC, which can lead to large conformational changes in the complementarity determining regions of the TCR to gain new contacts (25).

We also have demonstrated that borrowing the surface of an endogenous protein can reduce the affinity of interactions of a phosphopeptide with its SH2 domain. Steric hindrance or electrostatic repulsion are probably the basis for the decreased stability of the SH2 domain–SLFpYEEI–FKBP12 complex. In general, the creation of unfavorable contacts should be easier to achieve than favorable contacts, and it can be exploited to

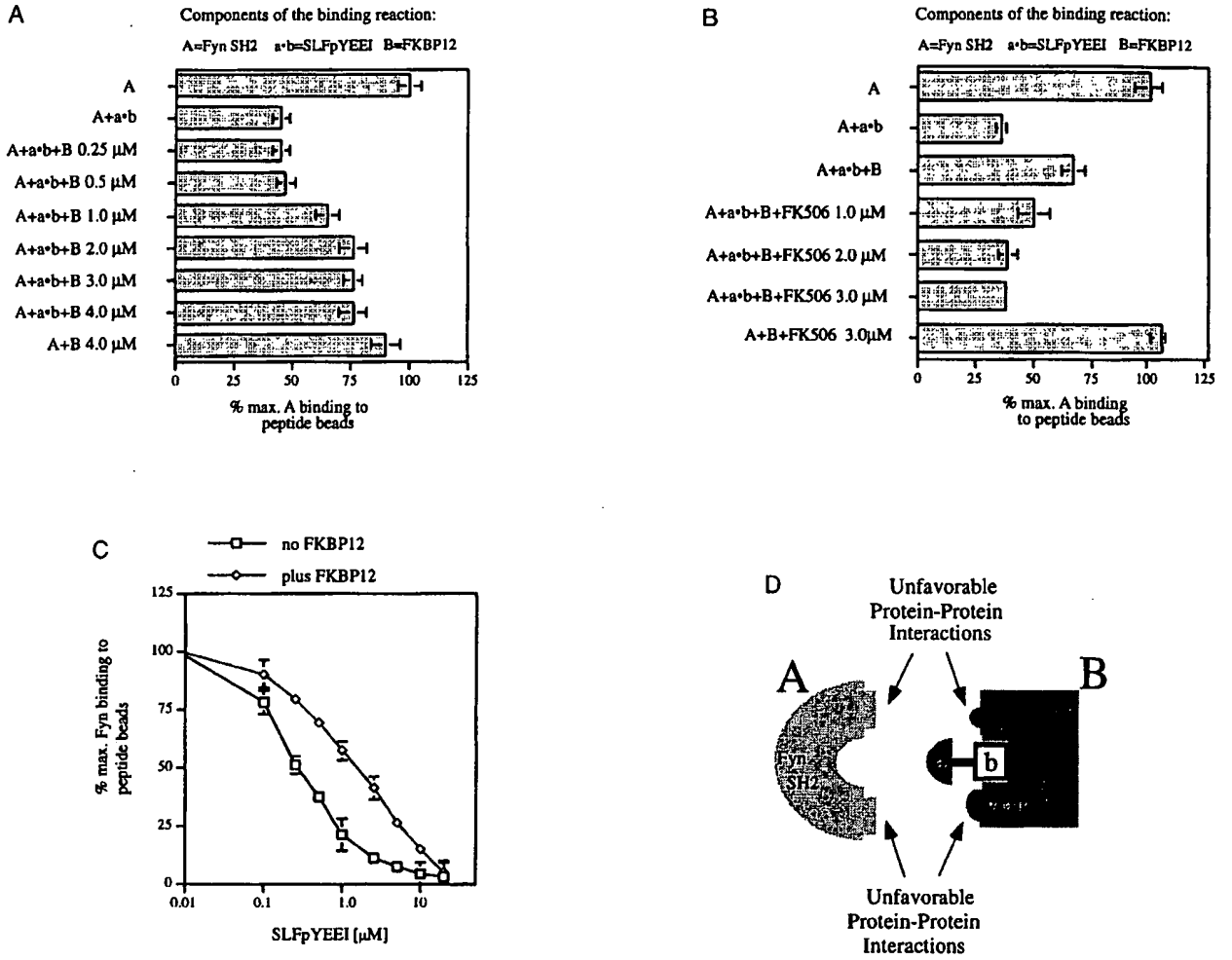

FIG. 4. Fyn SH2 domain binding to SLFpYEEI in a complex with FKBP12. (*A–C*) In 100 μl of binding buffer, peptide beads (7.5 μl) were incubated with the ^{32}P-labeled Fyn SH2 domain (200 nM, 18,000 cpm) and the molecules stated below. After a 2-hr incubation time, the beads were centrifuged and the radioactive Fyn SH2 domain bound was quantitated. (*A*) Addition of SLFpYEEI (1.0 μM) and increasing concentrations of FKBP12 (0.25–4.0 μM). (*B*) Addition of SLFpYEEI (1.0 μM), FKBP12 (2.0 μM), and increasing concentrations of FK506 (1.0–3.0 μM). The maximal radioactivity associated with the beads (=100%) was 12,583 cpm for *A* and 12,187 cpm for *B*. (*C*) Competition binding curves: ^{32}P-labeled Fyn SH2 domain (200 nM, 13,000 cpm) was incubated without or with FKBP12 (20 μM) in 100 μl of binding buffer. Increasing concentrations of FKpYEEI (0.1–20.0 μM) were added to the binding reactions. The maximal radioactivity associated with the beads was 9,284 cpm (=100%) for the ^{32}P-labeled Fyn SH2 domain alone and 10,492 cpm (=100%) for the ^{32}P-labeled Fyn SH2 domain in the presence of FKBP12. All data points were taken in triplicate, and the average was plotted. (Bars = SE.) (*D*) Model for unfavorable, destabilizing protein–protein interactions.

enhance the specificity of a molecule of interest. If, for example, a ligand binds to one desired and several undesired targets, a bifunctional molecule that causes unfavorable protein–protein interactions with the undesired targets may be selected. If the obtained bifunctional molecule shows favorable or at least neutral interactions with the desired molecule, specificity for the desired molecule will be created.

General Strategy to Modulate Ligand-Binding Affinities. The introduction of secondary binding interactions through synthetic modifications to small-molecule ligands has been examined in some detail (26, 27). Our approach differs by borrowing surface area from presenting proteins. Features of our strategy include the ability to (*i*) recruit different endogenous presenting proteins by changing one-half of the bifunctional molecule, (*ii*) vary the length and rigidity of the linker that joins the two halves of the bifunctional molecule, and (*iii*) vary the affinity of the small-molecule ligand that binds to the presenting protein. Changes to any of these three variables have the potential to directly affect the overall stability of the trimeric complex. Ultimately, this general strategy may prove useful to improve the affinity and/or specificity of small-molecule drugs for their targets. It can also provide a useful tool for the, so far, intractable problem of developing agonists or antagonists of protein–protein interactions.

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